

FORM PTO-1000
(REV. 10-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

534037mo

*U.S. APPLICATION NO. (if known, see 37 CFR 1.5)

INTERNATIONAL APPLICATION NO.
PCT/JP00/01494INTERNATIONAL FILING DATE
March 13, 200009/926138
PRIORITY DATE CLAIMED
March 18, 1999

TITLE OF INVENTION

ENZYMATIC FLUOROMETRIC ASSAY FOR cAMP AND ADENYLATE CYCLASE

APPLICANT(S) FOR DO/EO/US

Atsushi SUGIYAMA

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
 - (a) PCT Request in Japanese Language
 - (b) PCT/IB/304
 - (c) Demand
 - (d) Title page of WO 00/55356 together with International Search Report
 - (e) PCT/IB/308
 - (f) PCT/IB/332
 - (g) IPE Report (in Japanese)
 - (h) Formal drawings--four sheets

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

Neither international preliminary examination fee (37 CFR 1.482)
nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO
and International Search Report not prepared by the EPO or JPO \$1000.00

International preliminary examination fee (37 CFR 1.482) not paid to
USPTO but International Search Report prepared by the EPO or JPO. \$860.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but
international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)
but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)
and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT

CALCULATIONS PTO USE ONLY

\$ 860

Surcharge of **\$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).

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CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	27 - 20 =	7	X \$18.00
Independent claims	5 - 3 =	2	X \$80.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$270.00

TOTAL OF ABOVE CALCULATIONS	=	\$ 1,416
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§ 1,416

☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.

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SUBTOTAL = \$

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Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).

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TOTAL NATIONAL FEE	=	\$ 1,416
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\$ 1,416

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **\$40.00** per property +

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TOTAL FEES ENCLOSED = \$ 1,416

\$ 1,416

Amount to be refunded:	\$
charged:	\$

a. ☒ A check in the amount of \$ 1,416 to cover the above fees is enclosed. (Check No. 16276)

b. ☐ Please charge my Deposit Account No. _____ in the amount of \$_____ to cover the above fees.
A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 10-1213. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Eric S. Spector
JONES, TULLAR & COOPER, P.C.
P.O. Box 2266 Eads Station
Arlington, VA 22202

SIGNATURE: _____

ERIC S. SPECTOR

NAME _____

22495

REGISTRATION NUMBER

4/pds

DESCRIPTION

ENZYMATIC FLUOROMETRIC ASSAY FOR
cAMP AND ADENYLATE CYCLASE

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TECHNICAL FIELD

The present invention provides a method of determining cAMP content or an adenylate cyclase activity in a biological sample containing non-cyclic adenine nucleotides selected from the group consisting of cAMP produced from ATP by endogenous adenylate cyclase (cyclic adenosine-3',5'-monophosphate), ATP (adenosine-triphosphate), ADP (adenosine-diphosphate) and a mixture thereof without the use of radioactive agents. More particularly, the invention relates to a method which comprises: (1) combining a biological sample with effective amounts of apyrase, adenosine deaminase and alkaline phosphatase to enzymatically remove non-cyclic adenine nucleotides other than cAMP, and glucose-6-phosphate in the sample; (2) enzymatically converting cAMP into AMP; (3) determining an amount of AMP without the use of radioactive agents.

Adenylate cyclase (adenylyl cyclase, adenylate cyclase, EC4.6.1.1) is an enzyme catalyzing the conversion:



in the presence of Mg^{2+} or Mn^{2+} .

Adenylate cyclase exists locally on cell membranes and

plays a critical role as a signal transduction cascade of a number of fundamental hormones and neurotransmitters.

For example, measurement of adenylate cyclase activity has been employed to study the altered physiology exhibited by transplanted human hearts and in congestive heart failure. See M.R. Bristow et al., *New Engl. J. Med.*, 307, 205 (1982); K.G. Lurie et al., *J. Thorac. Cardiovasc. Surg.*, 86, 195 (1983).

Adenylate cyclase activity can be determined by monitoring the changes of cAMP content synthesized from ATP by the catalytic action of adenylate cyclase.

However, a more clear elucidation of the biological role of adenylate cyclase has been limited by the difficulty in monitoring accurately changes in the tissue level of cAMP.

cAMP (cyclic adenosine-3',5'-monophosphate) was found as a factor which intermediates blood sugar rising action of adrenaline and glucagon in liver cells. [E.W. Sutherland et al., *J. Am. Chem. Soc.*, 79, 3608 (1957)]. And also cAMP was found to intermediate actions of hormones such as adrenocorticotropin (ACTH), luteinizing hormone (LH), tyrosine stimulating hormone (TSH) and parathyroid hormone (PTH) or physiological active substances such as prostaglandin. Thus, when peptide hormones or active amines have been secreted and have reached at target cells,

cAMP transfers information for them to proceed enzymatic reactions, that is, plays a role of a second messenger.

cAMP is synthesized from ATP by adenylate cyclase located on membranes in the living body and decomposed by phosphodiesterase into 5'-AMP. cAMP is present widely in bacteria or animals but the concentration of cAMP is extremely low (a stationary concentration is 0.1 - 1 nmol/g wet weight). As an assay of cAMP, an assay using cAMP binding protein or radioimmunoassay is conveniently employed. The cAMP content depends on eutrophy, proliferation, differentiation, adaptation of cells and changes in sensitivity.

Measurement of cAMP in a wide variety of mammalian and non-mammalian tissue and fluids provides a useful way to assess cell viability, endocrine hormonal axis function, adenylate cyclase activity and phosphodiesterase activity. In addition, measurement of cAMP can be used to evaluate the activity of a number of signal transduction proteins, including , but not limited to, the family of G proteins (guanine-nucleotide binding protein) which play a major role in signal transduction, ribosomal protein synthesis, translocation of nascent proteins and other important cellular functions. Bourne et al., *Nature*, 348, 125 (1990).

Furthermore, measurement of cAMP may be used in evaluating other endogenous and exogenous compounds (for

example, nitrous oxide) which may alter the level of cyclic nucleotides in a particular cell, tissue, organ or body fluid.

Many hormones use cAMP as a second messenger including, but not limited to: epinephrine, norepinephrine, adrenocorticotropin (ACTH), vasopression, glucagon, thyroxine, and thyroid-stimulating and melanocyte-stimulating hormones which are some of the principle regulatory hormones/proteins in the living organism. The activity of all of these hormones and regulators can be measured in tissues, serum, body fluids, and in all cell cultures (cells and medium) using the method for cAMP of the present invention. Measurement of these hormones is performed in a wide variety of disease states where hormonal imbalance may lead to specific pathology.

Once a hormone or regulatory protein interacts with a specific receptor, the second messenger, in this case, cAMP, is produced through a cascade of biochemical events. The production of cAMP can also be specifically inhibited in some cases by hormones which use a decrease in cAMP as part of the specific hormonal signal-transduction pathway. The result of this regulatory protein or hormone and receptor interaction can be, but is not limited to, (1) an alteration in cell permeability secondary, for example, to changes in ion channels, (2) and alteration in the rate of

enzyme catalyzed reactions sensitive to the concentration of cAMP, and (3) an alteration in the rate of protein synthesis including the synthesis and degradation of other enzymes. a content of cAMP can be used to directly and indirectly monitor the consequences after interacting a hormone or regulatory protein with a receptor.

Specifically, cAMP can be measured in urine or blood for use as a marker for drug levels, like aminophylline or theophylline which stimulate the adrenergic nervous system by preventing the breakdown of endogenous cAMP. Measurement of cAMP in cell cultures can be used to assess specific hormones, regulatory protein and drugs where cAMP represents a vital link in the signal transduction process.

cAMP can also be used to assess cell viability and stability by studying cells in the absence or presence of a specific hormone or regulatory protein. For example, measurement of cAMP in liver cells (hepatocyte) by glucagon, can be used to assess hepatocyte viability. This may be useful, for example, in organ and/or cell transplantation, for example heart, liver, lung, kidney, pancreas, skin and brain cell transplantation.

Measurement of the responsiveness of cells from biopsy samples after activation by a wide variety of hormones, regulatory proteins and drug which either increase or decrease cellular cAMP levels, can be used as a way to

specifically assess cell function.

A specific clinical example is the use of cAMP measurement in cardiac biopsies to assess the responsiveness of myocardium. Cardiomyopathic heart cells do not respond with the same rise in cAMP content after β -adrenergic stimulation as normal heart cells. The diagnosis of the severity of the heart disease and the efficacy of some drugs, such as β -adrenergic blockers and angiotensin converting enzyme inhibitors, can be made comparing the responsiveness of biopsy samples from normal hearts to cardiomyopathic hearts. Measurement of basal and/or stimulated levels of adenylate cyclase activity or cAMP in blood cells can be used to guide therapy in such patients. In addition, release of cAMP either intracellularly or into the arterial or venous circulation can be used as an indicator of the response of an organ and/or tissue to a variety of different physiologic and nonphysiologic stresses such as ischemia, hypoxia, or drug or hormonal stimulation. Tissue or body fluid levels of cAMP can be measured in nearly ever mammalian cell or body fluid, including blood cells and platelets, with this approach. In some tissues, cAMP levels can be measured in response to specific stimulators as an index on oncogenicity and/or invasiveness, in the case of samples of potentially tumorous cells. In other cases, measurement of

cAMP can be used to determine the effectiveness of specific therapies which may alter cAMP synthesis or degradation.

As described above, cAMP plays an important role as a second messenger in information transfer in cells as well as has also various physiological functions. It is significant in fields of basic and clinical medicine to measure cAMP synthesized from ATP by a catalytic action of adenylate cyclase in order to determine activity of adenylate cyclase or elucidate behavior of cAMP.

BACKGROUND ART

Measurement of adenylate cyclase activity is carried out by quantitative determination of cAMP produced from ATP as a substrate. Methods for measurement of cAMP are grouped into two methods using as a substrate (1) labeled ATP and (2) non-labeled ATP.

In the method using labeled ATP as a substrate (1), using ATP labeled by a radioactive element, for example, [α - 32 P] ATP, as a substrate, and cAMP ([32 P] cAMP) generated from radioactively labeled ATP is separated and determined. See Y. Salomon et al., *Anal. Biochem.*, 58, 541 (1974; R. A. Johnson et al., *In Method in Enzymology*, 195, 3 (1991)). The method employs sequential affinity chromatography with ionic exchange resin and aluminum oxide columns for separation of [32 P] cAMP from [α - 32 P] ATP.

Although this method is sensitive, it relies upon dangerously and costly radioactively labeled compounds.

On the other hands, the methods using non-labeled ATP are classified into (1) radioimmunoassay wherein
5 radioactively labeled cAMP is subjected to antigen-antibody reaction competitively with anti-serum including cAMP generated from non-labeled ATP and then radioactivity of binding antibody is assayed to determine cAMP content, and
10 (2) protein-binding assay wherein radioactivity of ^3H -cAMP bound with cAMP-dependent protein kinase is measured using specific binding between cAMP-dependent protein kinase and cAMP. See A.G. Gilman et al., *Proc. Natl. Acad. Sci. USA*,
67, 305 (1970).

The method using the substrate non-labeled ATP can not
15 compensate decomposition of cAMP by cyclic nucleotide phosphodiesterase and therefore the method is not appropriate for samples including strong phosphodiesterase activity.

Given the safety and environmental concerns, the use
20 of radioactive materials should be avoided. A need exists for a highly sensitive non-radioactive assay to measure adenylate cyclase activity and cAMP as an index of adenylate cyclase activity.

However, it is very difficult to determine cAMP
25 without using radioactive compounds because of the

extremely low concentration of cAMP in most mammalian tissues. In addition, since non-cyclic adenine nucleotides such as AMP, ADP and ATP in a biological sample are present in several hundred to several hundred thousand times the concentration of cAMP and also those chemical structures are similar to the that of cAMP, they act as interfering substances in assay of cAMP. Particularly, ATP is present in one hundred million times the concentration of cAMP and therefore it is substantially impossible to exactly determine cAMP without complete removal of endogenous ATP.

On the other hand, cAMP is converted into AMP by action of phosphodiesterase. An assay for AMP without radioactive compounds has been disclosed. Lowry et al. have developed a sensitive assay based on the fluorescence of reduced pyridine nucleotide. See O.H. Lowry et al., *A Flexible System of Enzymatic Analysis*, Harcourt Brace Jovanovich, New York (1972); F.M. Matschinsky et al., *J. Histochem. Cytochem.*, 16, 29 (1968). The assay depends on it that absorbency of reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm is 0.617 per 0.1 mmol and an absolute concentration of NADPH is calculated on absorbency of a sample.

An assay for AMP is disclosed which depends upon the stimulatory effects of AMP on glycogen phosphorylase, the enzyme that converts glycogen into glucose-1-phosphate in

the presence of inorganic phosphate (P_i). See E. Helmrich et al., *Biochemistry*, 52, 647 (1964); *ibid.*, 51, 131 (1964); M. Trus et al., *Diabetes*, 29, 1 (1980). According to the method, glycogen phosphorylase activity is

5 determined by an amount of glucose-1-phosphate generated from glycogen and AMP can be assayed using the glycogen phosphorylase activity as an index. Lurie also have developed a sensitive assay for AMP. See K. Lurie et al., *Am. J. Physiol.*, 253, H662 (1987).

10 A method to increase the analytical sensitivity and specificity for cAMP or adenylate cyclase have employed enzymic degradation of non-cyclic adenine nucleotides or their removal by chromatography. See N.D. Goldberg et al., *Anal. Biochem.* 28, 523 (1969); B.Mcl. Breckenridge, *Proc. Natl. Acad. Sci. USA*, 52, 1580 (1964).

In the conventional analysis, since interfering endogenous ADP or ATP could not be completely removed, it has been considered that measurement of cAMP should be impossible. Therefore, there has been substantially no

20 method for highly sensitive measurement of cAMP content and adenylate cyclase activity based on the amount of cAMP without using radioactive substances.

From a completely different point of view, the inventor and others previously attempted to develop assays

25 for adenylate cyclase activity and cAMP and as a results of

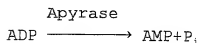
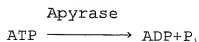
an intensive study, they had found a method for highly sensitive measurement of cAMP content and adenylate cyclase activity based on the amount of cAMP with using only enzymatic and chemical reactions, which comprises removing selectively interfering substances, endogenous non-cyclic adenine nucleotides, such as ATP, ADP and the like using enzymes, converting AMP into ATP, converting ATP into glucose-6-phosphate through fructose-6-phosphate, converting NADPH, determining NADPH concentration and correlating with cAMP concentration (WO94/17198).

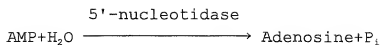
According to the method, cAMP at an amount of μg order in a biological sample can be strictly measured at a level of pmol or fmol . See A. Sugiyama et al., *Anal. Biochem.*, 218, 20 (1994); A. Sugiyama et al., *J. Clin. Lab.*, 8, 437 (1994); A. Sugiyama et al., *Anal. Biochem.*, 225, 368 (1995); A. Sugiyama et al., *Yamanashi Med. J.*, 10, 11 (1995).

The reaction schemes of the conventional method above are described below.

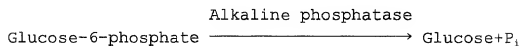
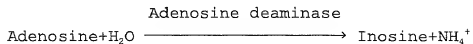
Stage 1: Cleaning Reactions

(Removal of endogenous non-cycle nucleotides and endogenous glucose-6-phosphate)



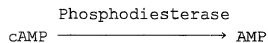


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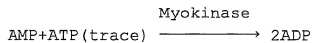
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Stage 2: Conversion Reaction 1
(Conversion of cAMP to AMP)

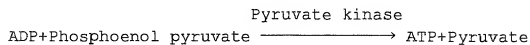


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Stage 3: Conversion Reactions 2
(Conversion of AMP to ATP)

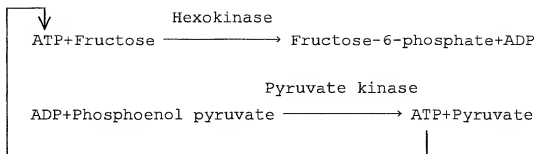


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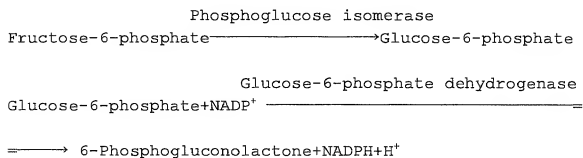
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Stage 4: Amplification by ATP-ADP Cycling Reactions
(Assay sensitivity is amplified by 6000 - 10000
times)



Stage 5: Detection Reactions

(Immunophotometric assay of NADPH)



The method as stated above was very excellent as a method for quantitative analysis in a principle of methodology and theoretically correct. However the method takes long time for a cleaning reaction or a reaction mixture gets cloudy when an enzyme has been deactivated by heating after a cycle reaction.

DISCLOSURE OF INVENTION

The present invention has been accomplished as a

result of intensive study for development of a simple and fast method for determining cAMP and adenylate cyclase without radioactive substances.

The present invention has improved on the methods of determining adenylate cyclase activity and quantitative analysis for cAMP with enzymatic reactions and fluorescence intensity which the inventor and others previously developed (WO94/17198). That is, (1) in Cleaning Reactions by deleting of 5'-nucleotidase from the enzymes to be used, 1 hour of the reaction period can be extremely shortened to from 5 to 10 minutes; (2) in Cycling Reactions, enzymes used are deactivated by removing Mg^{2+} with a chelating agent such as EDTA, instead of heating. The reaction mixture does not get cloudy and accuracy of Detecting Reaction on the next step is improved; (3) Converting Reaction is changed to 1 step reaction from conventional 2 steps converting reactions. The operation can be simpler; and (4) the concentrations of reaction agents used in Detecting Reactions were reviewed to optimize in the present method. By these improvements, an enzymatic fluorometric assay or a spectrophotometric assay in which cAMP and adenylate cyclase corresponding to cAMP are determined quickly and in high sensitivity could be provided.

Incidentally, as described below, the present method

can be used for measurement of guanine regulatory proteins and cAMP specific phosphodiesterase.

The reactions used in the present method for determining cAMP are shown below.

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Step 1 - Cleaning Reactions

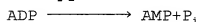
(Removal of endogenous non-cycle nucleotides and endogenous glucose-6-phosphate)

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Apyrase



Apyrase



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Alkaline phosphatase

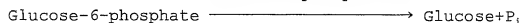


Adenosine deaminase

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Alkaline phosphatase



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Step 1 - Optional Cleaning Reactions 1

(Removal of fructose-6-phosphate)

Alkaline phosphatase



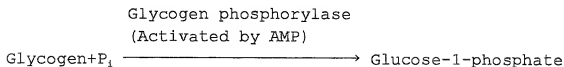
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Step 1 - Optional Cleaning Reactions 2

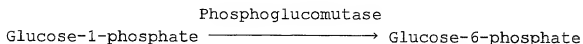
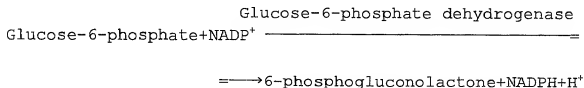
Step 3 - Detecting Reactions 1

(Fluorophotometric assay of NADPH)

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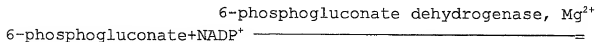
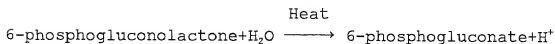
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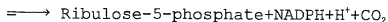
Step 3 - Optional Detecting Reactions

(Degradation of 6-phosphogluconolactone)

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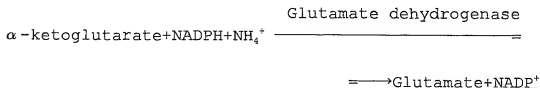


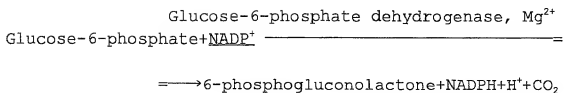
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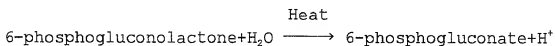
Step 3 - Detecting Cycling Reactions 1

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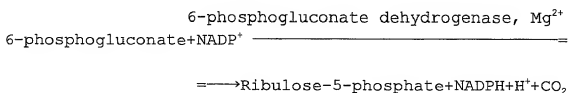




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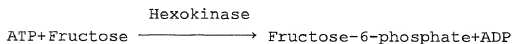
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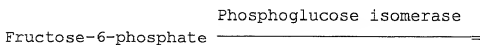
Step 3 - Detecting Reactions 2

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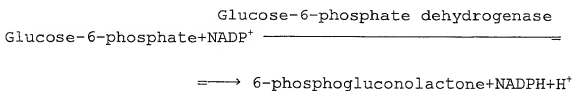
(Fluorophotometric assay of NADPH)



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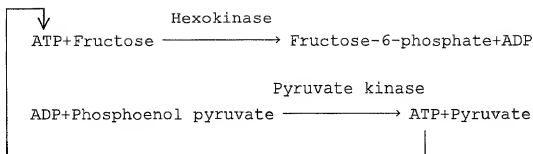


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Step 3 - Detecting Cycling Reactions 2

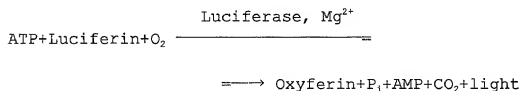
(Amplification by ATP-ADP cycling reaction)

(Assay sensitivity is amplified by 6000 - 10000 times)



Step 3 - Detecting Reactions 3

(Detection of ATP) (Luciferase reaction)



The reactions illustrated in the above schemes are further demonstrated below.

Step 1 - Cleaning Reactions

(Removal of endogenous non-cyclic adenine nucleotides and glucose-6-phosphate)

The present method for measurement comprises steps in which endogenous compounds having non-cyclic adenine group other than cAMP (adenosine, ATP, ADP and AMP) are enzymatically removed by a mixture of apylase, adenosine deaminase and alkaline phosphatase. Preferably, the

present method comprises a step in which glucose-6-phosphate in a sample is enzymatically converted into glucose by using alkaline phosphatase (Cleaning Reactions).

The Cleaning Reactions of the present invention can
5 remove all endogenous ATP, ADP and AMP which are present in much higher concentrations than cAMP and substantially increase the background signal. Since glucose-6-phosphate generates during subsequent Detecting Reactions, it is favorable to enzymatically remove glucose-6-phosphate from
10 a sample previously for improving the precision of measurement. The Cleaning Reactions are important for raising sensitivity.

Also, the Cleaning Reactions period was extremely shortened and simplified by deleting 5'-nucleotidase from
15 four kinds of enzymes, i.e. apyrase, 5'-nucleotidase, alkaline phosphatase and adenosine deaminase which have been used in a conventional cleaning reaction step. That is, it has been found that about 1 hour of the conventional cleaning reaction period can be shortened to only 5 to 10
20 minutes.

Step 1 - Optional cleaning reactions 1

(Removing of fructose-6-phosphate)

It is favorable to remove fructose-6-phosphate, which
25 is generated during Cycling Reactions and Detecting

Reactions, from a sample previously by hydrolysis with alkaline phosphatase.

Step 1 - Optional Cleaning Reactions 2

(Removal of endogenous glycogen in a sample)

It is more favorable to remove from a sample endogenous glycogen, which is converted to glucose-6-phosphate by using glucose oxidase, glycogen phosphorylase and alkaline phosphatase (Optional reactions). According to this Optional Cleaning Reactions, endogenous glycogen which is an interfering substance in Detecting Reactions, wherein a known amount of glycogen is added, is destroyed.

Step 2 - Converting reaction (Conversion to AMP)

Subsequently, phosphodiesterase is combined with the reaction mixture after the Cleaning Reactions so that cAMP is converted to AMP (Converting Reaction).

Step 3 - Detecting Reactions

(Fluorometric assay of NADPH)

After the Converting Reactions, glycogen and inorganic phosphoric acid are added to the reaction mixture and an amount of AMP is determined by correlating a level of glucose-6-phosphate which is finally generated from glycogen with glycogen phosphorylase activated by AMP.

Glucose-1-phosphate is converted to glucose-6-phosphate with phosphoglucomutase and finally glucose-6-phosphate is enzymatically converted into 6-phosphogluconolactone, NADPH and H^+ . The concentration of NADPH is measured by fluorometric assay, for example, according to the method of Trus et al. [M. Trus et al., *Diabetes*, 29, 1 (1980)].

Step 3 - Optional Detecting Reactions

(Degradation of 6-phosphogluconolactone)

And also, 6-phosphogluconolactone can be converted into 6-phosphogluconate by heating in an aqueous solution in vitro, and then 6-phosphogluconate can be converted into NADPH and ribulose-5-phosphate in the presence of $NADP^+$ in vitro. The concentration of NADPH can be increased by the Optional Reaction and measured by fluorometric assay, for example, according to the method of Trus et al. as described above [M. Trus et al., *Diabetes*, 29, 1 (1980)].

Where stimulated adenylate cyclase activity was measured in the same preparation from rabbit heart with both the modified Salomon radioactivity method and the present fluorometric method without alkaline phosphatase, the results were similar. Weign et al., *Anal. Biochem.*, 208, 217 (1993). Result with the radioactive assay are comparable to the present fluorometric method. Although the absolute specific activities are different when the

results from the radioactive assay and the fluorometric assay are compared, the fold stimulation of adenylate cyclase as determined using either method is similar. The differences in specific activities are most likely due to minor factors in adenylate cyclase reaction mixtures. That is, unlabelled cAMP is used in the radioactive assay to prevent [^{32}P]cAMP degradation by endogenous phosphodiesterases, whereas theophylline is used in the fluorometric assay to inhibit endogenous phosphodiesterase degradation of newly synthesized cAMP.

Moreover, measurement of adenylate cyclase using ATP-ADP Cycling reactions as shown in Step 3 - Detective Cycling Reactions 2, revealed that the absolute specific activities were the same for both the radioactive and fluorometric assays.

The method of the present invention is conveniently carried out by a kit previously prepared, and such a kit comprises vials containing enzymes, buffer solutions and the like to be used at each reaction step.

Specifically, a kit for performing a method for quickly determining the cAMP content or the adenylate cyclase activity of the present invention is exemplified by a kit which comprises (1) a vial for the cleaning reactions comprising effective amounts of apylase, alkaline phosphatase and adenosine deaminase to remove endogenous

non-cyclic adenine nucleotides consisting of ATP, ADP and AMP, and endogenous glucose-6-phosphate in a biological sample; (2) a vial for the converting reaction comprising an effective amount of phosphodiesterase to enzymatically convert cAMP in the biological sample to AMP; and (3) vials for the detecting reactions comprising effective amounts of (a) glycogen, inorganic phosphoric acid and glycogen phosphorylase to convert glycogen to glucose-1-phosphate, (b) phosphoglucomutase to convert glucose-1-phosphate to glucose-6-phosphate, and (c) glucose-6-phosphate dehydrogenase and NADP(β -nicotinamide adenine dinucleotide phosphate)* to convert glucose-6-phosphate to 6-phosphogluconolactone and NADPH.

Also, another kit for performing a method for quickly determining the cAMP content or the adenylate cyclase activity of the present invention may be a kit which comprises (1) a vial for the cleaning reactions comprising effective amounts of apylase, adenosine deaminase and alkaline phosphatase to enzymatically remove endogenous non-cyclic adenine nucleotides consisting of ATP, ADP and AMP, and endogenous glucose-6-phosphate in a biological sample; (2) a vial for the converting reaction comprising effective amounts of phosphodiesterase, ATP, myokinase, phosphoenol pyruvate and pyruvate kinase to enzymatically convert cAMP in the biological sample to AMP; and (3) vials

for the detecting reactions comprising effective amounts of
 (a) fructose and hexokinase to convert ATP to fructose-6-
 phosphate and (b) phosphoglucose isomerase, glucose-6-
 phosphate dehydrogenase and NADP^+ to convert fructose-6-
 5 phosphate to 6-phosphogluconolactone and NADPH.

The method of the present invention is sensitive
 enough to measure cAMP in small biological samples weighing
 less than 0.1 mg and can be adapted to measure 0.1 fmol
 cAMP/sample. According to the present invention, a known
 10 amount of ATP is added to a biological sample and then the
 ATP is converted into cAMP by action of adenylate cyclase
 in the sample. Adenylate cyclase activity may be provided
 by determining the converted AMP after removing all adenine
 nucleotides such as endogenous ATP by Cleaning Reactions.

15 Ammonium ion produced from adenosine in Cleaning
 Reaction drives the cleaning reaction sequence essentially
 to completion, preventing reformation of nucleotides in the
 sample. These steps provide for a significant unexpected
 improvement over fluorometric assays. Weign et al., *Anal.*
 20 *Biochem.*, 208, 217 (1993).

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 is a line graph showing the fluorescence
 against concentration of ATP in various incubation periods.

25 Figure 2 is a graphical plot of the absorbance against

concentration of standard samples of cAMP as determined by an embodiment of the invention.

Figure 3 is a histogram showing the amounts of cAMP detected in untreated and stimulated cells as determined by the present fluorometric method (solid) and commercially available immunocolorimetric assay (shaded) and radioimmunometric assay (clear).

Figure 4 is a histogram showing the adenylate cyclase activity determined by the present fluorometric method (solid) and commercially available radioimmunometric assay (shaded) and Salomon method (clear).

BEST MODE FOR CARRYING OUT THE INVENTION

The physiological material which is assayed with the present method is preferably obtained from a mammalian source, including tissue, blood cell, bone and physiological fluids such as urine, blood, spinal fluid and the like and may be fresh or frozen. Lowry et al., *A Flexible System of Enzymatic Analysis*, Harcourt Brace Jovanovich, New York (1972).

Thus, in a preferred embodiment, the present invention comprises the steps of:

Step 1 - Cleaning Reaction (Removal of endogenous non-cyclic nucleotides and glucose-6-

phosphate)

A sample of a physiological material comprising cAMP, glucose-6-phosphate and at least one non-cyclic adenine nucleotide, i.e., selected from the group consisting of ATP, ADP, AMP and mixture thereof is combined with an aqueous buffer comprising a mixture of apyrase, adenosine deaminase, and alkaline phosphatase so that said non-cyclic adenosine nucleotides (the ATP, ADP and/or AMP) are enzymatically converted to remove.

The wording "more quickly" means that the period of Cleaning Reaction which has conventionally taken about 1 hour is shorten within about 5 to 10 minutes, i.e., by from a twelfth to a sixth, at least a sixth of the reaction period.

Step 1 - Optional Cleaning Reactions 2

And optionally, the reaction mixture is combined with a mixture of glucose oxidase and glycogen phosphorylase and alkaline phosphatase so that all glycogen in the sample is destroyed, while the cAMP is retained in the reaction mixture. These steps destroy all endogenous glycogen which is an interfering substance in Step 3 - Detecting Reaction (Fluorometry of NADPH) in which a known amount of glycogen is added.

Step 2 - Converting Reactions (Reaction of conversion
to AMP)

The reaction mixture is combined with phosphodiesterase,
so that said cAMP is converted to AMP.

5

Step 3 - Detecting Reactions
(Fluorometric assay of NADPH)

Further said AMP is contacted with glycogen
phosphorylase in a presence of glycogen and inorganic
phosphatase so that glucose-1-phosphate is produced in the
reaction mixture. Then the glucose-1-phosphate is
enzymatically converted into 6-phosphogluconolactone, NADPH
and H^+ in said reaction mixture and the concentration of
NADPH is fluorometrically measured. The concentration of
NADPH is correlated with the concentration of adenylate
cyclase activity, cAMP content or a concentration of AMP.

The method of determining cAMP is based on a principle
that AMP produced by cleavage of 3',5'-phosphodiester bond
of cAMP stimulates glycogen phosphorylase activity. That
is, the amount of cAMP and adenylate cyclase activity
correspond to the absorbance of NADPH finally obtained from
glycogen phosphorylase activity which is activated by AMP
produced from cAMP.

The correlation of a final concentration of NADPH with
a concentration of AMP can be obtained, for example, by a

calibration curve. See Figure 2.

Preferably, following the Cleaning Reactions, the enzymes used in the Cleaning Reaction may be deactivated by heating.

5 Further preferably, following Step 1 - Cleaning Reactions, a solution of phosphodiesterase, glycogen-phosphorylase, glucose-1,6-diphosphate, inorganic phosphate, glycogen, NADP^+ , glucose-6-phosphate dehydrogenase, phosphoglucumutase and Mg^{2+} is added to the reaction
10 mixture and Steps 2 of Converting Reactions and Step 3 of Detecting Reactions 1 are carried out sequentially, *in situ*.

Step 3 - Optional Detecting Reactions 1

(Degradation of phosphogluconolactone)

15 The concentration of NADPH which is generated in Step 3 can be increased by sequentially converting the 6-phosphogluconolactone to 6-phosphogluconate by heating the reaction mixture of Step 3 - Detecting Reactions 1 and then reacting the 6-phosphogluconate with added NADP^+ and 6-phosphogluconate dehydrogenase in the presence of Mg^{2+} to
20 yield ribulose-5-phosphate, NADPH, H^+ and CO_2 .

Step 3 - Detective Cycling Reaction 1

The effective concentration of NADPH which is generated in Step 3 - Detecting Reactions 1 can be
25 increased in orders of magnitude by employing it in cycling

reaction system. One such reaction system converts NADPH added to α -ketoglutarate into NADP^+ and glutamate. The NADPH^+ in turn converts added glucose-6-phosphate into 6-phosphogluconolactone and NADPH. As described above, the

5 6-phosphogluconolactone can be hydrolyzed (H_2O , heat) and converted to ribulose-5-Phosphate and NADPH in the presence of 6-phosphogluconate dehydrogenase. Lowry et al., *A Flexible System of Enzymatic Analysis*, Harcourt Brace Jaccovanovich, New York (1972).

10 Step 2 - Optional Converting Reactions 1 (Reaction of Conversion to ATP)

Alternatively, the AMP which is produced from cAMP in Step 2 - Converting Reactions can be converted to ADP by combining it with a trace amount of ATP in the presence of

15 myokinase. The ADP which is produced is then converted to ATP and pyruvate by combining the ADP with 2-phospho(enol)pyruvate kinase. One molecular AMP produces one molecular ATP in this step (Reaction of conversion to ATP).

20 Step 2 - Converting Reactions and Step 2 - Optional Converting Reactions 1, in which cAMP is converted to AMP, and AMP is converted to ATP through ADP, can be carried out sequentially at one step.

25 Step 3 - Detecting Reactions 2

(Fluorometric assay of NADPH)

Further, ATP is converted to fructose-6-phosphate and ADP by combining with hexokinase in the presence of fructose. Fructose-6-phosphate is converted in the presence of phosphoglucosomerase to glucose-6-phosphate, which is enzymatically converted to 6-phosphogluconolactone, NADPH and H^+ . The concentration of NADPH is measured by the method of Trus et al. as described above. M. Trus et al., *Diabetes*, 29, 1 (1980).

Step 3 - Optional Detecting Reactions 1

Alternatively, as described above, the 6-phosphogluconolactone can be hydrolyzed and further converted to 6-phosphogluconate, which can be converted to NADPH, H^+ , CO_2 and ribulose-5-phosphate.

Step 3 - Detective Cycling Reactions 2 (Amplification of ATP-ADP cycling reaction)

Further, ATP is subjected to ATP-ADP cycling reaction. That is, ATP is converted to fructose-6-phosphate and ADP by combining it with hexokinase in the presence of fructose. Then the ADP is converted to ATP and pyruvate by combining phosphoenol pyruvate in the presence of pyruvate kinase. The ATP thus produced is again converted to fructose-6-phosphate and ADP. By repeating of these reactions, fructose-6-phosphate can be finally accumulated.

A chelating agent is added in order to deactivate

enzymes at the end of the Cycling Reactions. The chelating agents can be exemplified by polyaminocarboxylic acid such as EDTA, oxycarboxylic acid such as citric acid, preferably, EDTA.

5 The resulting fructose-6-phosphate, produced from the excess fructose and phospho(enol)pyruvate used in the Cycling Reactions, is converted to glucose-6-phosphate using phosphoglucose isomerase, and the glucose-6-phosphate is converted into 6-phosphogluconolactone and NADPH by exposing the glucose-6-phosphate dehydrogenase and NADP⁺.
10 The NADPH concentration can then be determined by Trus et al. method. M. Trus et al., *Diabetes*, 29, 1 (1980). See Step 3 - Detecting Reactions 2 as described above.

Step 3 - Detecting Reactions 3

15 According to the present invention, it is possible to absorptiometrically detect the ATP produced as shown in Step 2 - Optional Converting Reactions 1 with chemiluminescence assay that utilizes the luciferase reaction and firefly luciferin. Wulff et al., *Methods of Enzymatic Analysis*, Bergmeyer H.U., eds., VCH (1985). From
20 ATP determination, it is possible to calculate the AMP concentration and the cAMP concentration and adenylate cyclase activity corresponding to them.

25 Although the rate of this reaction is very slow, the yield of the reaction (defined as the ratio of the number

of emitted photons and the number of converted ATP molecules) is almost 100%. The intensity of the emitted light is directly proportional to the ATP concentration and is measured at 582 nm.

5 If a principle of a reaction system is identical, the present method of the present invention can be adapted to determination of any substance other than AMP, for example, guanylate cyclase activity, guanosine 3',5'-cyclic monophosphate (cGMP) and guanosine 3',5'-monophosphate (GMP) by applying a suitable enzyme corresponding to GMP. 10 The reaction schemes used are shown below.

Step (i) - Cleaning Reactions

Alkaline phosphatase



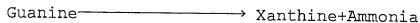
Alkaline phosphatase



Nucleoside phosphorylase



Guanase



25 Step (ii) - Converting Reactions

Phosphodiesterase

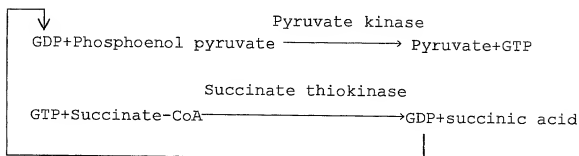


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Guaninemonophosphatekinase



Step (iii) - Cycling Reactions



10

Step (iv) - Detecting Reactions

Lactate dehydrogenase

15



Step (i) - Cleaning Reaction

As described above, a cleaning mixture of alkaline phosphatase, nucleoside phosphorylase and guanase is used

in the Cleaning Reactions. Alkaline phosphatase may be used to dephosphorylate compounds such as glucose-6-phosphate and non-cyclic nucleotides which may increase blank values during the measurement of cGMP (Cleaning Reactions).

In the Cleaning Reactions, GTP in a sample is converted to GMP plus 2Pi followed by conversion to guanosine and Pi. Guanosine is converted into guanine and ribose-1-phosphate and guanine is converted into xanthine and ammonia. Likewise in the Cleaning Reaction for determination of cAMP, the formation of ammonium (or NH_4^+) in the final Cleaning Reactions drives the series of linked reactions essentially to completion, ensures removal of the interfering nucleotides.

Preferably, the enzymes used in the Cleaning Reaction are deactivated, e.g., by heating, prior to the next Step (ii) - Converting Reaction.

Step (ii) - Converting Reaction

The cGMP present in the sample is then converted to GMP with phosphodiesterase. The GMP is combined with ATP in the presence of guanine monophosphate kinase to yield guanosine-5'-diphosphate (GDP) and ADP.

Step (iii) - Cycling Reactions

The GDP is employed in the presence of excess PEP, succinyl-CoA and inorganic phosphate (Pi) to yield an

amount of pyruvate (Cycling Reactions).

Step (iv) - Detecting Reactions

This pyruvate is quantitated indirectly by adding a known amount of NADH which in the presence of acid is converted to lactate and NAD^+ . Thus, the fluorescence of the indicator samples is decreased in direct proportion to the amount of pyruvate generated in the sample to be assayed for cGMP, GMP or guanylate cyclase. Also, in order to increase the assay sensitivity, after decomposition of excess NADH with acid, NAD^+ formed may be converted with alkali to 2-hydroxy-nicotinic aldehyde having higher fluorescence intensity. K. Scya et al., Anal. Biochem., 272, 243 (1999).

According to the present invention, an alternative way to measure GMP is to measure an amount of ATP which is decomposed by guanine monophosphate kinase in Step (ii) - Converting Reaction. The amount of ATP can be determined by a known method as described above.

In addition to improving assay sensitivity, measurement of adenylate cyclase activity or guanylate cyclase activity with the present enzymatic assays is significantly less costly, less time consuming, moreover, safer for the operator and better for environment because of using no radioactive substance.

Finally, the present assay can readily be adapted to

determine the amount of endogenous or exogenous phosphodiesterase in a biological sample. That is, a single, preselected amount of cAMP is added to a sample containing an unknown concentration of phosphodiesterase.

5 Any inhibitors specific to phosphodiesterases can be added as needed. A standard curve is generated by adding a single, preselected excess amount of cAMP to different preselcted, known amounts of phosphodiesterase. After a finite amount of reaction time (5-60 minutes) in which some
10 but not all of the added cAMP is transformed to AMP by the phosphodiesterase, the reaction is stopped. A cAMP standard curve can be run concurrently to verify that all reactions are working adequately. A cleaning reaction is initiated to degrade all non-cyclic adenine nucleotides.
15 The remaining cAMP will be inversely proportional to the native plus added phosphodiesterase. Then cAMP is converted to AMP by a conventional method to determine.

Specifically, a tissue sample is dissolved in a SET buffer (Sucrose 0.5 M, Tris 0.03 M, EDTA 2 mM) and to the
20 resultant mixture, the double volumes of PDE mixture (Tris 50 mM, cAMP 50 μ M) is added and incubated at 37 °C for 20 minutes. The reaction mixture is heated at 80 °C for 30 minutes. Cleaning Reaction Mixture in a volume four times as much as a tissue sample is added to the reaction mixture
25 and incubated at 37 °C for 1 hour. The reaction mixture is

heated at 80 °C for 30 minutes. Converting Reaction Mixture in a volume ten times as much as a tissue sample is added to the reaction mixture and incubated for 1 hour at room temperature. The fluorescence intensity of resultant NADPH is measured using Detecting Reactions.

The present invention provides a preparation comprising enzymes or buffers to be used in a form of kit. The reaction materials of each step can be conveniently prepared in a kit, for example, such as Cleaning Reaction Mixture kit, Converting Reaction Mixture kit, Cycling Reaction Mixture kit, Detecting Reaction Mixture kit.

The enzymes used in the present invention are provided in the form of, for example, aqueous solutions, lyophilized or solid formulations in a container such as a vial, an ample made by suitable glass or plastic.

The enzymes can be dissolved in buffers, electrolyte, distilled water, or they can be separately put in different containers and can be mixed with just before use.

Additives such as preservatives, stabilizers, dye stuffs, excipients, pH regulators and the like can be suitably added.

In addition to the amplification reaction of the present invention, the assay as described in the present invention can employ appropriate automatic analyzers.

The present invention provides a novel, non-radioactive enzymatic fluorometric assay for adenylate cyclase activity and cAMP, as well as cAMP specific phosphodiesterase activity and the biological activity of the G regulatory proteins. And also it provides a non-radioactive enzymatic fluorometric assay for guanylate cyclase activity and cGMP.

The method of the present invention offers several advantages over currently available methods compared to conventional methods for measurement of adenylate cyclase activity and the like. Unlike the assays previously disclosed by Y. Salomon et al., in *Anal. Biochem.*, 58, 541 (1974) and *Adv. Cyclic Nucleotide Res.*, 10, 35 (1979), the present assay does not utilize any radioactive material. In addition, this assay is more sensitive and simpler to perform than the previous assays.

And also, according to the present invention, the reaction period in the method can be extremely shortened and the operation becomes simpler compared to a method which previously developed by the inventor and others. See A. Sugiyama et al., *Anal. Biochem.*, 218, 20 (1994); A. Sugiyama et al., *J. Clin. Lab.*, 8, 437 (1994); A. Sugiyama et al., *Anal. Biochem.*, 225, 368 (1995); A. Sugiyama et al., *Yamanashi Med. J.*, 10, 11 (1995). In addition, a problem of cloudy in a sample, which is not avoidable in

conventional methods, is solved by using a chelating agent such as EDTA. And cAMP content in a biological sample in of order of μg can be correctly measured on a level of pmol or fmol .

5 The Cleaning Reaction of the present invention can remove all endogenous ATP, ADP and AMP which are present in a far higher concentration than cAMP and would otherwise substantially increase the blank. Additionally, the Cleaning Reactions can remove all endogenous glucose-6-
10 phosphate and glycogen as interfering substances in the sample. These Cleaning Reactions are important for improving assay sensitivity.

15 It has been found that the reaction period of 1 hour by conventional methods could be significantly shortened to about 5 - 10 minutes by deletion of 5'-nucleotidase from four enzymes, i.e., apyrase, 5'-nucleotidase, alkaline phosphatase and adenosine deaminase which have been used in the conventional cleaning reactions.

20 After Cycling Reactions, since Mg^{2+} is trapped by a chelating agent and excess of enzymes are deactivated without heating, the sample does not get cloudy.

25 The sensitivity of this assay can also be increased by reducing the reaction volumes. It can also be increased further by varying the concentrations of glycogen and inorganic phosphate in the reaction mixture, as reported by

Meinrich et al. and E. Helmrich et al., *Biochemistry*, 52, 647 (1964). According to the present invention, measurement of adenylate cyclase activity is possible in mammalian tissue biopsy samples comprising as little as 10.0 μ g of membrane protein.

Unlike the radioactive methods, where sensitivity is limited by the specific activity of $[\alpha\text{-}^{32}\text{P}]\text{cAMP}$ and the volume size for chromatographic separation and the like, there are no significant barriers to further increasing the sensitivity of the present fluorometric method. For example, cAMP has been measured over a broad concentration range of 1 fmol- 1 mmol.

EXAMPLES

The invention will be further described by reference to the following detailed examples wherein the enzymes, substrates and cofactors used were obtained from Boehringer Mannheim Co., except for apyrase and 5'-nucleotidase which were from Sigma Co., St. Louis, MO. The $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, $^3\text{H}\text{-cAMP}$, and Aquasol scintillation cocktail were purchased from New England Nuclear. Neutral Chromatographic Alumina WN-3 was obtained from Bio-Rad.

Example 1

Determination of Cleaning Reaction Period

A volume of 3 μL of ATP standard (0, 10, 100, 1000 and

10000 pmol/tube) was added into a 10x57 Pyrex[®] assay tube. While at room temperature, 25 μ L of Cleaning Reaction Mixture (100 mM Tris-HCl, pH 8.0; 2 mM MgCl₂; 2 U/mL apyrase; 10 U/mL adenosine deaminase; 40 U/mL alkaline phosphatase) was added to each assay tube. The mixture was incubated at 37 °C for 0, 5, 10, 15 and 20 minutes. Then Step 2 - Converting Reactions (Conversion to AMP), Optional Converting Reactions (Conversion to ATP), Step 3 - Detecting Cycling Reactions 2 were sequentially carried out and an fluorescence intensity at 340 nm was measured to determine an incubation period when ATP have completely disappeared. The results are shown in Figure 1. From the results, it was found that an interfering substance, i.e., ATP, substantially disappeared after at most 10 minutes of incubation.

Comparing Example

This test was carried out in order to compare Cleaning Reactions of the present invention with that of the cleaning reactions containing 5'-nucleotidase.

Preparation of a cleaning reaction mixture containing 5'-nucleotidase

Tris-HCl(pH 8.0), MgCl₂, 5'-nucleotidase, Apyrase, Adenosine deaminase and Alkaline phosphatase were mixed and to the mixture water was added up to total 25 μ L. The cleaning reaction mixture having final concentrations as

shown in Table 1 was prepared

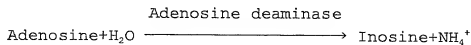
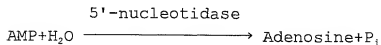
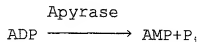
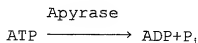
Table 1

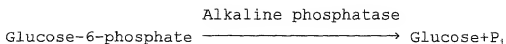
A cleaning reaction mixture containing 5'-nucleotidase

5

<u>Compound</u>	<u>Final Reaction Concentration</u>
Tris-HCl pH 8.0	100 mM
MgCl ₂	2 mM
5'-nucleotidase	2.5 U/mL
Apyrase	2 U/mL
Adenosine deaminase	10 U/mL
Alkaline phosphatase	20 U/mL
Water	up to a total volume 25 μ L

The cleaning reactions can be shown in the following reaction schemes.





Determination of the cleaning reaction period

Cleaning reactions were carried out using the cleaning reaction mixture prepared above as described in Example 1.

A volume of 3 μL of ATP standard (0, 10, 100, 1000 and 10000 pmol/tube) was added into a 10 \times 57 Pyrex[®] assay tube. While at room temperature, 25 μL of the cleaning reaction mixture (100 mM Tris-HCl, pH 8.0; 2 mM MgCl_2 ; 2 U/mL apyrase; 2.5 U/mL 5'-nucleotidase; 0.1 U/mL adenosine deaminase; 20 U/mL alkaline phosphatase) was added to each assay tube. The mixture was incubated at 37 $^{\circ}\text{C}$. Then Step 2 - Converting Reactions, Cycling Reactions and Detecting Reactions as described in Example 2 below were carried out and an absorbance at 340 nm was measured to determine an incubation period when ATP have completely disappeared. As the results, it was found that ATP had completely disappeared after about 1 hour of incubation.

Example 2

Micro cAMP measurement in Tissue Cultures with Enzymatic Fluorometric assay

(1) Preparation of Samples and Reaction Mixtures

A. Preparation of Biological samples

(Ventricular myocyte preparation)

Isolated ventricular myocytes were obtained from hearts of 1 day-old rats, grown in primary cultures. There were approximately 5 million viable myocardial cells per heart. After plating at a density of approximately 1 million cells per 100 mm, dish cells were grown in minimum essential medium with Hanks balanced salt solution containing 5% bovine calf serum cells. Simpson et al., *Cir. Res.*, 51, 787 (1982). On day 4, the medium was changed. The cultures contained >90% myocardial cells and cell numbers were constant over time. Simpson et al., *Cir. Res.*, 51, 787 (1982); Rocha-Singh et al., *J. Clin. Invest.*, 88, 204 (1991); and Rocha-Singh et al., *J. Clin. Invest.*, 88, 706 (1991)

Six plates of myocytes were randomized into two groups (n=3 plates/group). The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was added to the medium of each group to achieve a 2 μ M final concentration. After 5 minutes, isoproterenol was added to the stimulated group to achieve a final 1 μ M concentration, while the control group received no additional drug. After 5 minutes, cells from all six plates and plates of culture medium without cells were eluted with a total of 5 mL of 100% ethanol. One-tenth of the eluent (0.5 mL) from each dish was removed and air dried in a 12 x 75 mm borosilicate glass tube and

stored at -80°C . The other 4.5 mL were similarly air dried and stored. At the time of assay, the pellet was thawed and resuspended in 100 μL of 0.5 N perchloric acid. The extracts were stirred at 4°C for 2 minutes and sonicated for 1 minute using a sonicator (Branson Cleaning Equipment Co. A Smith-Kline Co., USA). The extract was neutralized with 25 μL of 2N KOH, centrifuged at 2000 g for 30 minutes and 80 μL of supernatant was removed for assay.

B. Preparation of Cleaning Reaction Mixture

(See Step 1 - Cleaning Reactions)

One molar Tris-HCl (pH 8.0) 400 μL ; 0.2 mM MgCl_2 40 μL ; 500 U/mL apyrase 32 μL ; 400 U/mL adenosine deaminase 100 μL ; 4000 U/mL alkaline phosphatase 4 μL were mixed and to the mixture water was added up to a total volume 4 mL to prepare Cleaning Reaction Mixture (Table 2)

Table 2 Cleaning Reaction Mixture

<u>Compound</u>	<u>Final Reaction Concentration</u>
Tris-HCl pH 8.0	100 mM
MgCl_2	2 mM
Apyrase	4 U/mL
Adenosine deaminase	10 U/mL
Alkaline phosphatase	40 U/mL
Water	up to a total volume 4 mL

After cooling rat ventricular myocyte preparation prepared in Example 2 (1) on ice, 0.4 μL of Reaction Mixture of Step 1 - Optional Cleaning Reaction (100 mM Tris-HCl, pH 8.0; 3 mM MgCl_2 3 U/mL apyrase; 150 $\mu\text{g/mL}$ adenosine deaminase; 30 U/mL alkaline phosphatase and 75 $\mu\text{g/mL}$ glycogen Phosphorylase- α) was added to the preparation to remove all endogenous adenine nucleotides, glycogen and glucose-6-phosphate. After incubation at 37 $^{\circ}\text{C}$ for 1 hour, enzymes were deactivated by heating at 80 $^{\circ}\text{C}$ for 30 minutes.

C. Preparation of Converting Reaction Mixture

(See Step 2 - Converting Reactions and Step 2 -

Optional Converting Reactions)

One molar Tris-HCl (pH 8.0) 400 μL , 0.2 mM MgCl_2 40 μL , 4 % bovine Serum Albumin 10 μL , 1 M KCl 600 μL , 0.01 M dithiothreitol 80 μL , 0.1 μM ATP 16 μL , 0.5 M Phosphoenolpyruvate 24 μL , 2 U/mL Phosphodiesterase 24 μL , 720 U/mL Myokinase 24 μL and 2000 U/mL Pyruvate kinase 160 μL were mixed and to the mixture water was added up to a total volume 4 mL to prepare Converting Reaction Mixture (Table 3).

Table 3 Converting Reaction Mixture

<u>Compound</u>	<u>Final Reaction Concentration</u>
Tris-HCl pH 8.0	100 mM

	MgCl ₂	2 mM
	Bovine Serum Albumin	0.01 %
	KCl	150 mM
	Dithiothreitol	2 mM
5	ATP	40 nM
	Phosphoenolpyruvate	3 mM
	Phosphodiesterase	12 mU/mL
	Myokinase	4.5 U/mL
	Pyruvate kinase	80 U/mL
10	Water	up to a total volume 4 mL

D. Preparation of Cycling Reaction Mixture

(See Step 3 - Detective Cycling Reactions 2)

One molar Tris-HCl (pH 8.0) 400 μ L, 0.2 mM MgCl₂ 40 μ L,
 15 4 % bovine Serum Albumin 10 μ L, 0.1 M Fructose 30 μ L, 1500
 U/mL Hexokinase 160 μ L were mixed and to the mixture water
 was added up to a total volume 4 mL to prepare Cycling
 Reaction Mixture (Table 4).

20 Table 4 Cycling Reaction Mixture

	<u>Compound</u>	<u>Final Reaction Concentration</u>
	Tris-HCl pH 8.0	100 mM
	MgCl ₂	2 mM
	Bovine Serum Albumin	0.01 %
25	Fructose	3 mM

Hexokinase 60 mM
 Water up to a total volume 4 mL

E. Preparation of Detecting Reaction Mixture

(See Step 3 - Detecting Reaction 2)

One molar Tris-HCl (pH 8.0) 2000 μ L, 0.2 mM EDTA 320 μ L, 0.1 M NADP⁺ 120 μ L, 3500 U/mL Phosphoglucose isomerase 6 μ L, 1750 U/mL Glucose-6-phosphate dehydrogenase 6 μ L were mixed and to the mixture water was added up to a total volume 4 mL to prepare Cycling Reaction Mixture (Table 5).

Table 5 Detecting Reaction Mixture

<u>Compound</u>	<u>Final Reaction Concentration</u>
Tris-HCl pH 8.0	100 mM
EDTA	3.2 mM
NADP ⁺	0.01 %
Phosphoglucose isomerase	1 U/mL
Glucose-6-phosphate dehydrogenase	0.5 U/mL
Water	up to a total volume 4 mL

(2) Micro cAMP measurement in Tissue Cultures with Enzymatic Fluorometric assay

According to Enzymatic Fluorometric assay comprising a series of Cleaning Reactions, Converting Reactions, Cycling Reactions and Detecting Reactions, cAMP in the ventricular

myocyte preparation prepared in (A) as a sample was measured.]

1) Cleaning Reactions

(Step 1 - Cleaning Reactions)

5 A volume of 3 μ L of neutralized myocyte extract (either 2.4 % or 0.24 % of total eluent from 100 mm plate) or 3 μ L of ATP standard (0, 3.6, 7.2, 14.4, 21.6 and 28.8 pmol/20 μ L) was added into a 10 \times 57 Pyrex[®] assay tube (Iwaki glass Co.). While at room temperature, 25 μ L of Cleaning Reaction Mixture prepared from (B) (100 mM Tris-HCl, pH 8.0; 2 mM MgCl₂; 2 U/mL apyrase; 10 U/mL adenosine deaminase; 40 U/mL alkaline phosphatase) was added to each assay tube. The mixture was incubated at 37 °C for 10 minutes. Enzymes were then deactivated by heating for 30 minutes at 90 °C. 15 An internal tissue blank control was similarly prepared.

2) Converting Reactions

(Step 2 - Converting Reactions (conversion to AMP) and

- Optional converting Reactions (conversion to ATP))

20 A volume of 25 μ L of the Converting Reaction Mixture prepared from (C) (100 mM Tris-HCl, pH 8.0; 2 mM MgCl₂; 0.01 % bovine Serum Albumin; 150 mM KCl; 2 mM dithiothreitol; 40 nM ATP; 3 mM phosphoenol-pyruvate; 12 U/mL phosphodiesterase; 4.5 U/mL myokinase; and 80 U/mL 25 Pyruvate kinase) was added to each assay tube from Cleaning

Reactions. The mixture was incubated at room temperature overnight. The reactions were terminated by heating the assay tubes at 90 °C for 5 minutes.

3) Cycling Reactions

5 (Step 3 - Detecting Cycling Reactions 2)

A volume of 25 μ L of the Cycling Reaction Mixture prepared from (D) (100 mM Tris-HCl (pH 8.0); 2 mM $MgCl_2$; 0.01 % bovine Serum Albumin; 3 mM Fructose; 60 U/mL Hexokinase) was added to each assay tube from Converting Reactions. In view of the high concentrations of enzymes per reaction, it was important to set up these reactions at 0 °C to insure the same starting time for all assay tubes. The mixture was incubated at 37 °C for 2 hours (about 4000 - 10000 cycles).

15 4) Detecting Reactions

(Step 3 - Detecting Reactions 2)

A volume of 125 μ L of the Detecting Reaction Mixture prepared from (E) (100 mM Tris-HCl, pH 8.0; 3.2 mM EDTA; 0.6 mM $NADP^+$; 1 U/mL Phosphoglucose isomerase; 0.5 U/mL Glucose-6-phosphate dehydrogenase) was added to each assay tube from Cycling Reactions. After 20 minutes at room temperature, the final concentration of NADPH was measured using a fluorometer (Optical Technology Devises, Inc., NY). The fluorometer was set such that a reading of 10 fluorometric units was equivalent to 1 nmol of NADPH in 900

μL of buffer (50 mM Tris-HCl, pH 8.0).

Individual assay steps were verified by concurrently assaying internal controls using known concentrations of the appropriate substrate, for example, additional ATP was used to check Cleaning Reactions, an AMP standard curve was used to assess Converting Reactions and an ATP standard curve was used to assess Cycling Reactions.)

5) Results

Measurement of the absorbance from cAMP standard (0, 3.6, 7.2, 14.4, 21.6 and 28.8 pmol/20 μL) at 340 nm afforded the cAMP standard curve shown in Figure 2.

cAMP values of control group received no additional drug and isoproterenol-stimulated cells from (1) (A) were measured for one-tenth of the eluent from each plate of cells using the cAMP standard curve (Figure 2) to give an actual value of cAMP 680 pmol/100 mm plate.

The results of measurement of cAMP content using the fluorometric assay of the present invention, immunocolorimetric assay (Amersham International Co.) and radioimmunoassay (Amersham International Co.) were shown in Figure 3. The data generated from measurements of cAMP are sufficiently coincident with the data obtained from a commercially available radioactive assay with variability usually less than 5 %.

In addition, adenylate cyclase activity were measured

from the results of quantitative measurement of cAMP using the assay of the present invention, radioimmunoassay and Salomon method. The data from three methods were shown in Figure 4. The values of adenylate cyclase activity are assessed by cAMP amounts (pmol) per minute generated from protein 1 mg.

Example 3

Each appropriate amount of 1 M Tris-HCl pH 8.0, 0.2 mM $MgCl_2$, 1 M K_2HPO_4 , 0.1 mM AMP, 4000 U/mL alkaline phosphatase, and 10 mg/mL glycogen phosphorylase were mixed and to the mixture water is added to prepare Cleaning Reaction Mixture in final concentrations as shown below.

Table 6 Cleaning Reaction Mixture

<u>Compound</u>	<u>Final Reaction Concentration</u>
Tris-HCl pH 8.0	100 mM
$MgCl_2$	2 mM
K_2HPO_4	5 mM
AMP	0.1 mM
Alkaline phosphatase	20 U/mL
Glycogen phosphorylase	10 μ g/mL
Water	-

Each appropriate amount of 1 M Tris-HCl (pH 8.0), 0.1

mM MgCl_2 , 1 M K_2HPO_4 , 0.1 M AMP, 0.1 M dithiothreitol, 1 mM glucose-1,6-diphosphate, 4 % bovine Serum Albumin, 0.1 M NADP, and 10 mg/mL Glycogen phosphorylase, 10 mg/mL phosphoglucose mutase, and 5 mg/mL Glucose-6-phosphate dehydrogenase were mixed and to the mixture water is added to prepare Converting Reaction Mixture in final concentrations as shown below.

Table 7 Converting Reaction Mixture

<u>Compound</u>	<u>Final Reaction Concentration</u>
Tris-HCl pH 8.0	100 mM
MgCl_2	2 mM
K_2HPO_4	5 mM
AMP	0.1 mM
Dithiothreitol	1 mM
Glucose-1,6-diphosphate	4 μM
Bovine Serum Albumin	0.01 %
NADP	0.2 mM
Glycogen phosphorylase	20 $\mu\text{g/mL}$
Phosphoglucose mutase	4 $\mu\text{g/mL}$
Glucose-6-phosphate dehydrogenase	2 $\mu\text{g/mL}$
Water	-

1) Cleaning Reactions

(Step 1 - Optional Cleaning Reactions 2)

An aqueous glycogen solution comprising 206 μM of glycogen was prepared and distributed into 12 Pyrex® assay tubes (Iwaki Glass Co., 10×57 mm), each three tubes in an amount of 0, 20, 40 and 80 μL , and water was added to each assay tube up to a total volume 80 μL . At room temperature, 500 μL of Cleaning Reaction Mixture (100 mM Tris-HCl, pH 8.0; 2 mM MgCl_2 ; 5 mM K_2HPO_4 ; 0.1 mM AMP; 20 U/mL Alkaline phosphatase; and 10 $\mu\text{g/mL}$ Glycogen phosphorylase) was added to each assay tube. The mixture were incubated at 37 °C for 60 minutes.

2) Detecting Reactions

(Step 3 - Detecting Reactions 1)

A volume of 500 μL of the converting Reaction Mixture (100 mM Tris-HCl, pH 8.0; 2 mM MgCl_2 ; 5 mM K_2HPO_4 ; 0.1 mM AMP; 1 mM dithiothreitol, 4 μM glucose-1,6-diphosphate, 0.01 % bovine Serum Albumin, 0.2 mM NADP, and 20 $\mu\text{g/mL}$ glycogen phosphorylase, 4 $\mu\text{g/mL}$ phosphoglucumutase and 2 $\mu\text{g/mL}$ glucose-6-phosphate dehydrogenase) was added to each assay tube. The mixture was incubated at 37 °C for 30 minutes. The fluorescence wavelength of 460 nm for each assay tube was measured at a exiting wavelength of 340 nm. In result, the data confirmed that glycogen in the glycogen solution was completely removed by the Cleaning Reactions.

Preventive effect against clouding by chelate agents

A volume of 125 μ L of Detecting Reaction Mixture without EDTA (100 mM Tris-HCl, pH 8.0; 0.6 M NADP⁺; 1 U/mL Phosphoglucose isomerase, 0.5 U/mL Glucose-6-phosphate dehydrogenase) was added to the assay tube after Cycling Reactions in Example 2. The mixture was incubated at 90 °C for 30 minutes and the enzymes were deactivated. Clouding in the assay solution was surveyed with eyes. The solution from Example 2 to which was added Detecting Mixture with EDTA was employed as a control. The enzymes in the control were deactivated by EDTA at room temperature.

In result, while the control was clear, the mixture deactivated by heating got cloudy.

INDUSTRIAL APPLICABILITY

The present invention provides a method of determining cAMP content or an adenylate cyclase activity in a biological sample without the use of radioactive agents.

That is, the cleaning reactions according to the present invention can remove selectively and efficiently endogenous non-cyclic adenine nucleotides such as ATP, ADP and AMP, and glucose-6-phosphate, which act as interfering substances in assay of cAMP. After conversion of cAMP in a sample into AMP, AMP is enzymatically converted into ATP.

ATP is then converted via fructose-6-phosphate into

glucose-6-phosphate in order to obtain NADPH, the concentration of NADPH is finally determined by a fluorometric method. By the correlation with a final concentration of NADPH, a cAMP content or adenylate cyclase activity can be safely obtained only by enzymatic or chemical reactions without the use of radioactive agents.

In particular, according to the present invention, the enzymes used in the cleaning reactions are limited to apyrase, alkaline phosphatase and adenosine phosphatase. Also, by adjustment of their concentrations, the present invention succeeds in making the processes for determination become much simpler and in making their reaction periods be extremely shortened within about 5 to 10 minutes, i.e., by from a twelfth to a sixth compared to conventional methods.

Since the cAMP content depends on eutrophy, proliferation, differentiation, adaptation of cells and changes in sensitivity, measurement of cAMP provides a useful way to assess cell viability, endocrine hormonal axis function, adenylate cyclase activity and phosphodiesterase activity. Therefore, the value of cAMP content can be an excellent index in the fields of basic and clinical medicine.

According to the method of the present invention, cAMP content and adenylate cyclase activity corresponding to

CLAIMS

1. A method of removing more quickly non-cyclic adenine nucleotides consisting of endogenous ATP, ADP and
5 AMP, and endogenous glucose-6-phosphate in a biological sample which comprises treating said sample with effective amounts of apyrase, alkaline phosphatase and adenosine deaminase to remove said non-cyclic adenine nucleotide and glucose-6-phosphate.

2. A method of determining cAMP content or an adenylate cyclase activity in a biological sample comprising the following steps:

Cleaning Reaction: combining a biological sample with
15 effective amounts of apyrase, alkaline phosphatase and adenosine deaminase to remove non-cyclic adenine nucleotides consisting of endogenous ATP, ADP and AMP, and endogenous glucose-6-phosphate;

20 Converting Reaction: enzymatically converting cAMP in the biological sample into AMP; and

Detecting Reaction: determining an amount of AMP without the use of radioactive agents.

3. The method according to claim 2 wherein, further
25 comprising, in said Cleaning Reaction, combining said

biological sample with effective amounts of glucose oxydase, glycogen phosphorylase and alkaline phosphatase so as to enzymatically remove endogenous glycogen from said biological sample.

5

4. The method according to claim 2 wherein said Converting Reaction is carried out by combining said biological sample with an effective amount of phosphodiesterase.

10

5. The method according to claim 2 wherein an enzyme used in said Converting Reaction of cAMP into AMP is deactivated by a chelating agent after conversion to AMP.

15

6. The method according to claim 5 wherein said chelating agent is EDTA.

20

7. The method according to claim 2 wherein said Detecting Reaction comprises conversion from glycogen to glucose-1-phosphate by contacting glycogen phosphorylase with glycogen in the presence of inorganic phosphoric acid added to said sample and said conversion is activated *in vitro* by said AMP.

25

8. The method according to claim 7 wherein said

Detecting Reaction further comprises combining said sample with an effective amount of phosphoglucomutase to convert glucose-1-phosphate into glucose-6-phosphate and then combining said sample with effective amounts of glucose-6-phosphate dehydrogenase to convert glucose-6-phosphate into 6-phosphogluconolactone and NADP^+ so as to convert glucose-1-phosphate into 6-phosphogluconolactone and NADPH.

9. The method according to claim 8 wherein said Detecting Reaction further comprises heating up said sample in the presence of water to convert 6-phosphogluconolactone into 6-phosphogluconate and then combining the sample with an effective amount of NADP^+ to convert 6-phosphogluconate into ribulose-5-phosphate and NADPH.

10. A method of determining cAMP content or an adenylate cyclase activity in a biological sample comprising the following steps:

Cleaning Reaction: combining a biological sample with effective amounts of apyrase, alkaline phosphatase and adenosine deaminase to enzymatically remove endogenous non-cyclic adenine nucleotides other than cAMP, and endogenous glucose-6-phosphate;

Converting Reaction: enzymatically converting cAMP in the biological sample into AMP; and

Detecting Reaction: enzymatically converting ATP into fructose-6-phosphate which is then enzymatically converted into 6-phosphogluconolactone and NADPH and determining a concentration of NADPH without the use of radioactive agents.

11. The method according to claim 10 further comprising, in said cleaning reaction, combining said biological sample with effective amounts of glucose oxydase, glycogen phosphorylase and alkaline phosphatase so as to enzymatically remove endogenous glycogen from said biological sample.

12. The method according to claims 10 or 11 wherein said Detecting Reaction comprises, after enzymatically converting fructose-6-phosphate into 6-phosphogluconolactone and NADPH, further heating the reaction mixture and then adding 6-phosphogluconate dehydrogenase and NADP^+ so as to convert said 6-phosphogluconolactone into ribulose-5-phosphate and NADPH.

13. The method according to claim 10 wherein in said Cleaning Reaction, endogenous non-cyclic adenine nucleotides other than cAMP is one or more of ATP, ADP, AMP and a mixture thereof.

14. The method according to claim 10 wherein, in said
Converting Reaction, the conversion of cATP into ATP is
carried out by a combination of effective amounts of
5 phosphodiesterase, myokinase and pyruvate kinase.

15. The method according to claim 10 wherein, in said
Detecting Reaction, the conversion of ATP into fructose-6-
phosphate is carried out by a combination of hexokinase and
pyruvate kinase.

16. The method according to claim 10 wherein, in said
Detecting Reaction, the conversion of fructose-6-phosphate
into 6-phosphogluconolactone and NADPH is carried out by
combination of phosphoglucose isomerase and glucose-6-
15 phosphate dehydrogenase.

17. The method according to claim 10 wherein, in said
detecting reaction, an enzyme for conversion of ATP into
20 fructose-6-phosphate is deactivated by a chelating agent
after conversion of non-cyclic adenine nucleotides.

18. The method according to claim 17 wherein said
chelating agent is EDTA.

19. The method according to claims 1, 2 or 10 wherein said biological sample is a mammalian tissue.

20. The method according to claims 1, 2 or 10 wherein
5 said biological sample is a physiological fluid.

21. A kit for determining cAMP content or an adenylate cyclase activity in a biological sample which comprises:

10 (1) a vial for Cleaning Reaction comprising effective amounts of apyrase, alkaline phosphatase and adenosine deaminase to remove non-cyclic adenine nucleotides consisting of endogenous ATP, ADP and AMP, and endogenous
15 glucose-6-phosphate in a biological sample;

(2) a vial for Converting Reaction comprising an effective amount of phosphodiesterase to enzymatically convert cAMP in a biological sample into AMP; and

20 (3) a vial for Detecting Reaction comprising glycogen, inorganic phosphoric acid and glycogen phosphorylase to convert glycogen into glucose-1-phosphoric acid; phosphoglucomutase to convert glucose-1-phosphate into glucose-6-phosphate; and glucose-6-phosphate dehydrogenase and NADP⁺ to convert glucose-6-phosphate into 6-phosphogluconolactone and NADPH.

22. A kit for determining cAMP content or an adenylate cyclase activity in a biological sample which comprises:

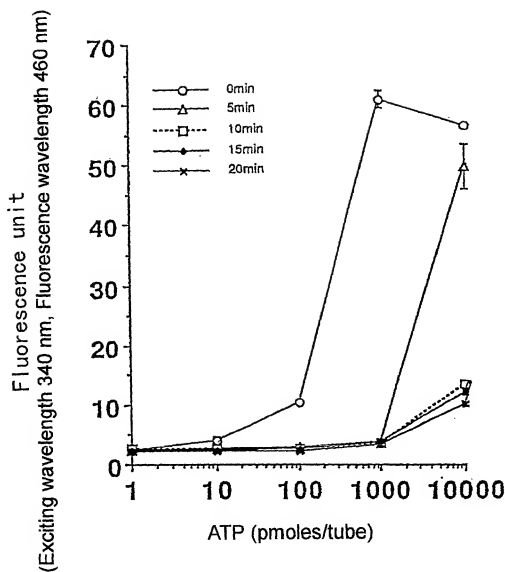
(1) a vial for cleaning Reaction comprising effective
5 amounts of apyrase, alkaline phosphatase and adenosine
deaminase to enzymatically remove endogenous non-cyclic
adenine nucleotides other than cAMP, and endogenous
glucose-6-phosphate in a biological sample;

(2) a vial for Converting Reaction comprising
10 effective amounts of phosphodiesterase, ATP, myokinase,
phosphoenolpyruvic acid and pyruvate kinase to
enzymatically convert cAMP in the biological sample into
ATP; and

(3) a vial for Detecting Reaction comprising fructose
15 and hexokinase to convert ATP into fructose-6-phosphate;
phosphoglucose isomerase, glucose-6-phosphate dehydrogenase
and NADP⁺ to convert fructose-6-phosphate into 6-
phosphogluconolactone and NADPH.

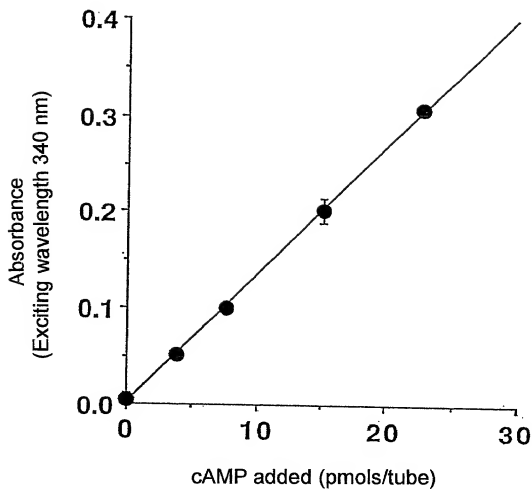
1 / 4

Fig. 1



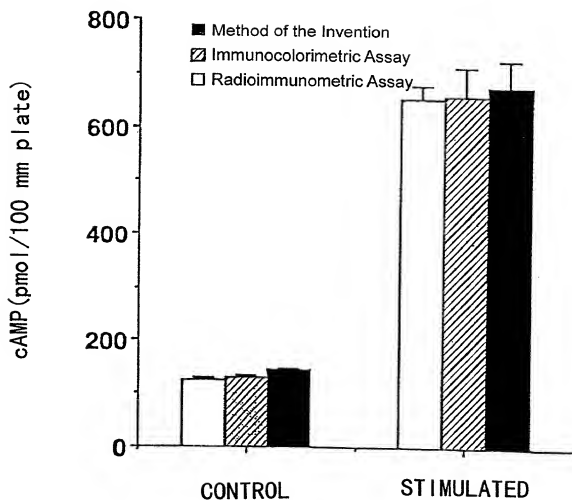
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Fig. 2



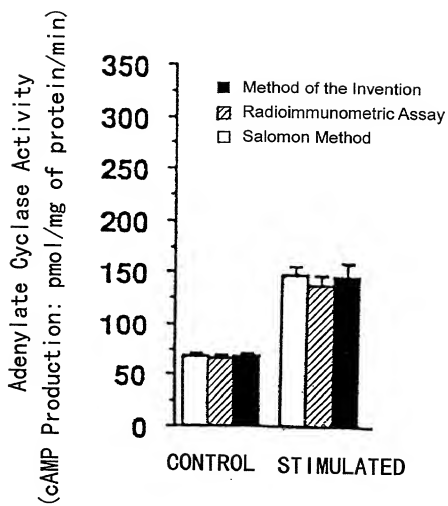
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Fig. 3



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Fig. 4



COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

This declaration is of the following type:

- ☒ original
- ☐ design
- ☐ supplemental
- ☐ national stage of PCT
- ☐ divisional
- ☐ continuation
- ☐ continuation-in-part (CIP)

My residence, post office address and citizenship are as stated next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed for and for which a patent is sought on the invention entitled:

ENZYMATIC FLUOROMETRIC ASSAY FOR CAMP AND ADENYLATE CYCLASE

the specification of which

- ☐ is attached hereto
- ☐ was filed on _____, as
Application Serial No. _____
and was amended on _____
(if applicable)
- ☒ was described and claimed in PCT International application
No. PCT/JP00/01494 filed on March 13, 2000
and as amended under PCT Article 19 on _____
(if any).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any Amendment referred to above.

I acknowledge duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Sec. 1.56.

- ☐ In compliance with this duty there is attached an information disclosure statement. 37 CFR 1.97.

I hereby claim foreign priority benefits under Title 35, United States Code, Sec. 119, of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent of inventor's certificate having a filing date before that of the application on which priority is claimed:

[] no such applications have been filed
[X] such applications have been filed as follows.

Prior Foreign Application(s)

<u>073690/1999</u>	<u>Japan</u>	<u>18.03.1999</u>	[X]	[]
(Number)	(Country)	(day/month/year filed)	Yes	No
<u> </u>	<u> </u>	<u> </u>	[]	[]
(Number)	(Country)	(day/month/year filed)	Yes	No

I hereby claim the benefit under Title 35, United States Code, Sec. 120 of any United States application(s) listed below, and insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Sec. 112, I acknowledge the duty to disclose all information known to be material to patentability as defined in Title 37, Code of Federal Regulations, Sec. 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

<u>(Application Serial No.)</u>	<u>(Filing Date)</u>	<u>(patented, pending, abandoned)</u>
<u>(Application Serial No.)</u>	<u>(Filing Date)</u>	<u>(patented, pending, abandoned)</u>

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Full name of third joint inventor, if any _____

Inventor's signature _____ (Date)

Residence _____

Citizenship _____

Post Office Address _____

Full name of fourth joint inventor, if any _____

Inventor's signature _____ (Date)

Residence _____

Citizenship _____

Post Office Address _____